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<b>(54) Title:</b> A METHOD OF PRODUCING TRANSFORMED CELLS OF BARLEY  <b>(57) Abstract</b> <p>An object of the present invention is to provide a method for efficient transformation of barley cells by improving a method of producing transformed plants with a microorganism belonging to the genus <i>Agrobacterium</i> conventionally used in transforming rice plants. The present method of producing transformed cells of barley comprises the steps consisting of: 1) suspending, in a suspension medium, a microorganism belonging to the genus <i>Agrobacterium</i> having a transformation ability with a foreign gene and containing said foreign gene; 2) culturing, in a co-culture medium, the microorganism belonging to the genus <i>Agrobacterium</i> suspended in the suspension medium and barley cells with calluses induced in a callus induction medium; 3) separating the cultured barley cells from the co-culture medium; and 4) placing the separated barley cells on a selective medium to select the transformed cells into which the foreign gene has been introduced. A further object of the present invention is the use of high concentrations of acetosyringone (&gt; 200 mg/l) in steps 1) and 2).</p>		

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## A METHOD OF PRODUCING TRANSFORMED CELLS OF BARLEY

### Field of Technology

The present invention relates to a method for transformation of barley cells.

### Background Technology

Known major methods of producing transformed plants such as barley (Hordeum) from the Gramineae family include a method of direct gene transfer into protoplasts (Funatsuki et al., 1995, Theor. Appl. Genet., 91:707-712) and a method using a particle gun (Wan and Lemaux, 1994, Plant Physiol., 104:37-48).

In the protoplast method, however, there remain many aspects to be improved with respect to the efficiency of production of transformed plants, applicable varieties etc. On the other hand, in the particle gun method there remain many aspects to be improved with respect to necessity for a special device as well as applicable varieties etc., like the protoplast method.

Recently, it has been revealed that a transformation method using microorganisms belonging to the genus Agrobacterium is effective in transformation of cereals such as rice (Chan et al., 1993, Plant Mol. Biol., 22:491-506; Hiei et al., 1994, Plant J., 6: 271-282).

This method using the Agrobacterium does not require any technique for plant regeneration from protoplasts or any special

device such as particle gun and is thus considered to be a very effective transformation method. For barley, however, production of transgenic plants with microorganisms belonging to the genus Agrobacterium is only one example (Tingay et al., 1997, Plant J., 11:1369-1376), and establishment of a transformation system for barley by microorganisms belonging to the genus Agrobacterium is desired.

#### Disclosure of the Invention

An object of the present invention is to provide a method of efficiently transforming barley cells by improving the above-mentioned method of producing transgenic plants by use of microorganisms belonging to the genus Agrobacterium which was utilised in transformation of rice plants (Chan et al., 1993, Plant Mol. Biol., 22:491-506; Hiei et al., 1994, Plant J., 6:271-282).

As a result of their eager study under these circumstances, the present inventors found that in the case where barley cells are transformed with a microorganism belonging to the genus Agrobacterium, barley cells can be transformed efficiently by co-culturing the barley cells with a microorganism belonging to the genus Agrobacterium in the presence of acetosyringone at a significantly higher concentration than in transformation of rice plants and for a longer period of time under lower temperature conditions than usual, and the present inventors thereby arrived at the present invention.

The first aspect of the present invention is a method of producing transformed cells of barley, comprising the steps consisting

of :

- 1) suspending, in a suspension medium, a microorganism belonging to the genus Agrobacterium having a transformation ability with a foreign gene and containing said foreign gene;
  - 2) culturing, in a co-culture medium, the microorganism belonging to the genus Agrobacterium suspended in said suspension medium and barley cells with calluses induced on a callus induction medium;
  - 3) separating the cultured barley cells from the co-culture medium;
- and
- 4) placing the separated barley cells on a selective medium to select the transformed cells into which the gene has been introduced.

The second aspect of the present invention is a production method according to claim 1 wherein the suspension medium contains 200 to 1000 mg/l acetosyringone.

The third aspect of the present invention is a production method according to the above method 1 or 2 wherein the co-culture medium contains 200 to 1000 mg/l acetosyringone.

The fourth aspect of the present invention is a production method according to any one of the above methods 1 to 3 wherein the culture in the co-culture medium is carried out at 20 to 24°C for 5 to 7 days.

#### Preferable Embodiments of the Invention

Hereinafter, the present invention is described by reference to preferred embodiments.

First, calluses with a high differentiation ability are induced

from barley tissues. Although the material used here is not particularly limited, immature embryos of barley 8 to 20 days after pollination are preferably used.

As the callus induction medium, use is made of an agar medium containing auxins such as IAA (indole acetic acid), 2,4-D (2,4-dichlorophenoxy acetic acid), picrolam, dicamba etc. Induction of calluses is carried out by culturing immature embryos of barley at 20 to 30°C for about 3 days after placed on said medium.

Although the strain of the microorganism belonging to the genus Agrobacterium is not particularly limited, a strain carrying a reporter gene such as GUS gene or the like is preferably used to confirm expression of a gene introduced into cells. A typical strain is Agrobacterium tumefaciens.

Suspension cells of this microorganism belonging to the genus Agrobacterium are then prepared. The microorganism belonging to the genus Agrobacterium cultured on the agar medium is transferred to and suspended in an acetosyringone-containing liquid medium (referred to hereinafter as suspension medium).

A gene can be introduced into barley cells more efficiently at a higher concentration of acetosyringone in the suspension medium where a concentration of 200 to 1000 mg/L is suitably used for the present invention.

The microorganism belonging to the genus Agrobacterium and barley cells with calluses induced on the callus induction medium, both suspended in the suspension medium, are co-cultured. That is, barley cells are immersed in the suspension medium at a regulated

concentration and then co-cultured on said agar medium containing acetosyringone.

As a result of examination of the concentration of acetosyringone in the co-culture medium, it was found that a particularly higher concentration than the usual concentration (10 mg/L) used for plants such as rice is preferable in the case of barley, and introduction of a foreign gene into barley cells is particularly effective where acetosyringone is used at a concentration of 200 to 1000 mg/L.

With respect to other conditions for co-culture, the period of culture is preferably a longer period such as 5 to 7 days. The period of co-culture of barley cells in conventional methods ranges from 2 to 3 days, but introduction of a foreign gene into the barley cells is inadequate. The culture temperature is preferably a lower temperature than the conventional temperature range of 25 to 28°C used for plants such as rice, so that growth of the microorganism belonging to the genus Agrobacterium can be inhibited and the period of co-culture can be prolonged, and the temperature range of 20 to 24 °C is found to be preferable for introduction of a foreign gene into barley cells.

Then, the barley cells thus co-cultured with the microorganism belonging to the genus Agrobacterium is washed with carbenicillin, cephotatis etc. and then placed on a selective medium containing antibiotics such as kanamycin, hygromycin etc. to permit selection of barley cells having the gene introduced into them.

While the transformed barley cells are selected, transient

assays are conducted to confirm introduction of the gene into the cells. The method of such transient assays is varied depending on the type of the gene introduced, and in the case where the GUS gene is introduced, expression of the introduced gene can be easily confirmed by the GUS assay (Jefferson et al., 1987, EMBO J., 6: 3901-3907).

According to the present invention, barley cells can be transformed efficiently with a microorganism belonging to the genus Agrobacterium. Once regenerated plants are obtained from the resulting transformed cells, it is also possible to produce transformed barley. The transformation method of the present invention does not require any technique of plant regeneration from protoplasts or any special device such as particle gun, and is thus a very practical method.

#### Examples

Hereinafter, the present invention is described in more detail by reference to Examples which however are not intended to limit the scope of the present invention.

##### Example 1

Immature embryos (10 days after pollination) from the barley variety Lenins grown in a field was placed in an MSm2D solid medium prepared by adding 30 g/L maltose, 2 mg/L 2,4-D and 2 g/L Gel Light to an MSm medium (Jaehna et al., 1991, Theor. Appl. Genet., 82:74-80), and calluses were induced (pre-cultured) therefrom in a bright place at 25°C for 3 days.

Separately, an EHA101 (pIG121Hm) strain i.e. one strain of



Agrobacterium tumefaciens was used as the microorganism belonging to the genus Agrobacterium. The EHA101 strain is described in Hood, E. E., G. L. Helmer, R. T. Fraley, M. D. Chilton, 1986, J. Bacteriol., 1291-1301, and plasmid pIG121Hm is described in Ohta, S., S. Mita, T. Hattori, K. Nakamura, 1990, Plant Cell Physiol., 31:805-813. This plasmid pIG121Hm is a kanamycin- and hygromycin-resistant vector containing the GUS gene.

The above strain from a glycerol stock was plated on a solid medium containing 50 mg/L kanamycin and 50 mg/L hygromycin and incubated in the dark at 28°C for 3 days. This was scratched off with a sterilized spatula and suspended in the MSm2D liquid medium (referred to hereinafter as suspension medium) prepared by adding 30 g/L maltose, 2 mg/L 2,4-D and a predetermined amount of acetosyringone to the MSm medium. The density of the microorganism was adjusted to 0.15-0.2 in terms of OD 600 nm.

Shoots and roots were removed from the immature embryos pre-cultured for 3 days, and then immersed for 90 seconds in the suspension medium. Excess water was removed therefrom with a sterilized paper towel, and the embryos were placed in the above MSm2D co-culture medium containing 10 g/L glucose and a predetermined amount of acetosyringone, and co-cultured for 3 days.

The immature embryos thus co-cultured were washed sufficiently with solution containing 500 mg/L carbenicillin. Thereafter, excess water was removed therefrom with a sterilized paper towel and placed on the MSm2D selection medium containing 50 mg/L hygromycin and 500 mg/L carbenicillin.

3 days later, introduction of the GUS gene into barley cells and its expression were examined in a transient test using GUS assays.

The results are shown in Table 1.

Table 1. Effect of acetosyringone concentration in GUS assays

Acetosyringone concentration (mg/L)		A	B (%)	D
in the suspension medium	in the co-culture medium			
10	10	98	8 (8.2)	3.1
	200	84	3 (3.6)	2.3
	1000	43	0 (0.0)	—
200	10	112	3 (2.7)	2.0
	200	114	3 (2.6)	4.3
	1000	114	4 (3.5)	40.8
1000	10	70	2 (2.9)	4.0
	200	52	6 (11.5)	9.5
	1000	27	3 (11.1)	32.7

A: The number of immature embryos placed in the co-culture medium.

B: The number of immature embryos which showed blue spot formation, indicating expression level of the introduced gene.

C: (The number of immature embryos which showed blue spot formation/the number of immature embryos placed in the co-culture medium) × 100

D: The mean number of blue spots formed in each immature embryo which showed blue spot formation.

As is evident from the table, the mean number of formed blue spots per immature embryo is higher where the concentration of acetosyringone in the suspension medium is higher, indicating effective introduction of the gene into barley cells. It was further

revealed that the gene is introduced effectively into barley cells where the concentration of acetosyringone in the co-culture medium is 200 to 1000 mg/l.:

#### Example 2

In this example, the culture period and temperature conditions in co-culture were examined in the same manner as in Example 1 except that the culture period used was 3 days or 6 days and co-culture was conducted at predetermined temperatures. Introduction of the GUS gene into barley cells and its expression were examined in the transient test using GUS assays. The results are shown in Tables 2 and 3.

Table 2. Effect of temperature and acetosyringone concentration for co-culture in GUS assays  
(period of co-culture: 3 days).

Temperature (°C)	Acetosyringone concentration (mg/L)	A	B(%)	D
20	10	30	0 (0.0)	—
	200	29	2 (6.9)	6.0
	1000	29	0 (0.0)	—
24	10	31	3 (9.7)	3.3
	200	27	3 (11.1)	1.3
	1000	30	3 (10.0)	4.0
28	10	31	3 (9.7)	9.0
	200	30	3 (10.0)	19.3
	1000	30	1 (3.3)	6.0

A: The number of immature embryos placed in the co-culture medium.

B: The number of immature embryos which showed blue spot formation, indicating expression level of the introduced gene.

C: (The number of immature embryos which showed blue spot formation/the number of immature embryos placed in the co-culture medium) $\times 100$

D: The mean number of blue spots formed in each immature embryo which showed blue spot formation.

Table 3. Effect of temperature and acetosyringone concentration for co-culture in GUS assays  
(period of co-culture: 6 days).

Temperature (°C)	Acetosyringone concentration (mg/L)	A	B(%)	D
20	10	16	0 (0.0)	—
	200	15	4 (26.7)	2.3
	1000	15	2 (13.3)	16.0
24	10	15	2 (13.3)	8.5
	200	14	3 (21.4)	8.0
	1000	15	1 (6.7)	121.0

A: The number of immature embryos placed in the co-culture medium.

B: The number of immature embryos which showed blue spot formation, indicating expression level of the introduced gene.

C: (The number of immature embryos which showed blue spot formation/the number of immature embryos placed in the co-culture medium) $\times 100$

D: The mean number of blue spots formed in each immature embryo which showed blue spot formation.

As is evident from these tables, the effect of introducing the gene into barley cells is higher when the culture period is longer and the temperature is 20 to 24°C.

### CLAIMS

1. A method of producing transformed cells of barley, comprising the steps consisting of :

- 1) suspending, in a suspension medium, a microorganism belonging to the genus Agrobacterium having a transformation ability with a foreign gene and containing said foreign gene;
- 2) culturing, in a co-culture medium, the microorganism belonging to the genus Agrobacterium suspended in the suspension medium and barley cells with calluses induced on a callus induction medium;
- 3) separating the cultured barley cells from the co-culture medium; and
- 4) placing the separated barley cells on a selective medium to select the transformed cells into which the foreign gene has been introduced.

2. A production method according to claim 1 wherein the suspension medium contains 200 to 1000 mg/l acetosyringone.

3. A production method according to claim 1 or 2 wherein the co-culture medium contains 200 to 1000 mg/l acetosyringone.

4. A production method according to any one of claims 1 to 3 wherein the culture in the co-culture medium is carried out at 20 to 24°C for 5 to 7 days.

# INTERNATIONAL SEARCH REPORT

Inter.      nal Application No

PCT/JP 98/03026

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 6    A01H1/04      A01H4/00

According to International Patent Classification(IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 6    A01H    C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 604 662 A (JAPAN TOBACCO INC) 6 July 1994	1
A	see page 3, line 52 - line 53 see page 4, line 19 - line 43 see page 6, line 2 - line 17 see page 8, line 12; example 1 see page 8, line 24 - line 25 see page 8, line 46 ----	2-4
X	EP 0 672 752 A (JAPAN TOBACCO INC) 20 September 1995	1
A	see page 6, line 9 see page 6, line 16 - line 39 see page 10, line 45 - line 55 see page 11, line 4 - line 25 ----- -/--	2-4

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>RHODORA, R.: "Agrobacterium tumefaciens-mediated transformation of japonica and indica rice varieties" PLANTA, no. 199, 1996, pages 612-617, XP002079226 see page 613, right-hand column, last paragraph see page 614; table 1</p> <p>---</p>	2-4
A	<p>GODWIN, I. ET AL.: "The effects of acetosyringone and pH on Agrobacterium-mediated transformation vary according to plant species" PLANT CELL REPORTS, no. 9, 1991, pages 671-675, XP002079227 see abstract; table 1 see page 674, right-hand column, paragraph 3</p> <p>-----</p>	2-4

# INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/JP 98/03026

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0604662 A	06-07-1994	AU 667939 B	18-04-1996
		AU 4513493 A	31-01-1994
		CA 2121545 A	07-01-1995
		US 5591616 A	07-01-1997
		WO 9400977 A	20-01-1994
		JP 2649287 B	03-09-1997
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EP 0672752 A	20-09-1995	AU 687863 B	05-03-1998
		AU 7546094 A	22-03-1995
		CA 2148499 A	09-03-1995
		WO 9506722 A	09-03-1995
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